Development of Methods For Quantitative Determination of Polyphenols in Grass *Pentaphylloides Fruticosa* (L.) O. Schwarz.

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Abstract—The purpose of this study was to develop a method of qualitative and quantitative assessment of biologically active substances of *P. fruticosa* L. herb. As object of study was used *P. fruticosa* L. herb. We used the method of high performance liquid chromatography in reversed-phase variant. As a result of chromatography, it was found that the chromatographic system used can effectively separate and identify the polyphenol complex of the herb *P. fruticosus* L. The chromatogram shows that the polyphenol complex of the herb *P. fruticosus* L. consists of the glycosides quercetin, kaempferol, rhamnetin, procyanidins, ellagotannins and ellagic acid and chlorogenic acid. Aglycones were found in the hydrolysis products: quercetin, kempferol, ellagic acid, and trace amounts of rhamnetin. Thus, as a result of this study, the composition of the polyphenolic complex of the herb *P. fruticosus* L. was established, including the glycosides of quercetin, kaempferol, rhamnetin, proacyanidins, ellagic acid and ellagotannins. A method has been developed for the quantitative determination of the sum of aglycones of grass polyphenols of *P. fruticosus* L., which is based on the principle of extraction with the simultaneous hydrolysis of glycosides of polyphenols to aglycones and their subsequent chromatographic determination by high performance liquid chromatography and quantification using absolute calibration.

Keywords—P. fruticosa L. herb, RP HPLC, quercetin glycosides, kaempferol glycosides, rhamnetin glycosides, proacyanidins, elagic acid, ellagotannins

I. INTRODUCTION

The creation of effective and safe drugs based on biologically active compounds of plant origin, designed to increase the body's resistance to pathogenic factors, is one of the dominant tasks of modern medicine. Interest in plant antioxidants is primarily due to the fact that this antioxidant activity is able to stop oxidative stress, which is the cause of many diseases of the cardiovascular, nervous, respiratory and other systems of the body. One of the plant sources with high antioxidant properties is the Kuril bush tea – *Pentaphylloides fruticosa* (L.) O. Schwarz. This plant contains large amounts of flavonoids and oxy-cinnamic acids, such as hyperoside, catechin, caffeine and ferulic acid, rutin and ellagic acid, glycosides of kaempferol [1-7]. Numerous studies have shown that extracts from the grass and flowers of plants have powerful antioxidant, antiradical, antimicrobial, antiviral, hypoglycemic, anti-inflammatory, antiulcerogenic activity [8-15].

There is a lot of information about the chemical and pharmacological study of *P. fruticosa* L., both in domestic and foreign literature [16-21]. However, the existing data are fragmentary and contradictory information that does not allow the use of the plant as a Pharmacopoeia. In addition, the study of the domestic pharmaceutical market of pharmaceuticals showed the absence of drugs based on this plant. Therefore, the qualitative and quantitative assessment of biologically active substances of the plant is an urgent problem.

The lack of clear information about the chemical composition, regulatory documents governing the quality control of the plant, determined the purpose of this study.

The purpose of this study was to develop a method of qualitative and quantitative assessment of biologically active substances of *P. fruticosa* L. herb.

The study of this plant is carried out as part of the development of a new scientific direction "Pharmaceutical remake" [22].

II. EXPERIMENTAL

As object of study was used *P. fruticosa* L. herb. We used the method of high performance liquid chromatography in reversed-phase variant. On the firm's chromatograph «*AgilentTechnologies 1200 Infinity*». Chromatographic column – *Ascentis express* C_{18}*2,7μm × 100 mm × 4,6 mm*.

HPLC water and ethyl alcohol (according to GOST R 51652) were used as the mobile phase, formic acid served as the acid modifier.

Calculation of the number of theoretical plates, separating the ability of the chromatographic system-separation coefficient R_s, and asymmetry of the chromatographic peak-asymmetry coefficient was calculated according to the European Pharmacopoeia.

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The polyphenol complex was chromatographed under the following conditions:

mobile phase speed – 0.5 ml/min;

column thermostat temperature +35 °C;

diode-matrix detection: 365, 375 nm

open sample volume – 5 µl.

The gradient elution regime was carried out under the conditions specified in table I.

The component composition was identified by matching the retention times of analytes with standard samples, as well as the results of diode-matrix detection.

The relative content of individual components was determined by internal normalization.

III. RESULTS AND DISCUSSION

To develop a method of identification and quantitative determination of the active components of *P. fruticosus* L. herb, a chromatographic system for the possibility of separation and identification of the polyphenol complex of *P. fruticosus* L. herb was tested at the first stage. For this purpose, the extraction of the corresponding raw materials with ethyl alcohol in 70% was carried out. The resulting extraction was chromatographed under the conditions given above.

The chromatogram of 70% ethanol extraction from the herb *P. fruticosus* L. is shown in fig.1.

As a result of chromatography, it was found that the chromatographic system used can effectively separate and identify the polyphenol complex of the herb *P. fruticosus* L. The chromatogram shows that the polyphenol complex of the herb *P. fruticosus* L. consists of the glycosides quercetin, kaempferol, rhamnetin, procyanidins, ellagotannins and ellagic acid and chlorogenic acid.

TABLE I. CONDITIONS OF GRADIENT ELUTION OF POLYPHENOLS P. FRUTICOSUS L.

Time, min	A,%	B,%
0	90	10
10	80	20
20	70	30
30	50	50
40	10	90

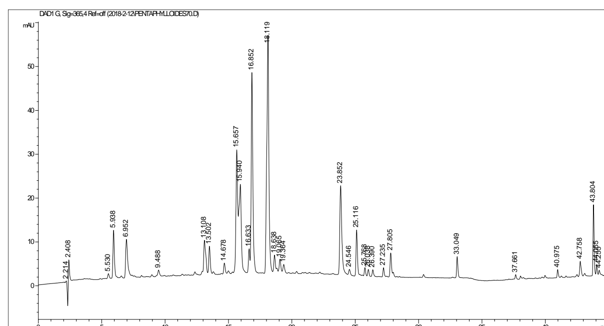

Fig.1. Chromatogram of 70% alcohol extraction from grass *P. fruticosa* L.

Such a variety of components makes it difficult to optimally quantify the raw material. Therefore we decided to develop a quantitative determination of the components of the raw materials at the contents aglycones. Since the dominant components of the herb *P. fruticosa* L. are flavonoids-glycosides of quercetin, kaempferol and ellagic acid, then we proposed to evaluate the quantitative content in terms of aglycones of these components.

The calculation of the content of these components was carried out by absolute calibration. For this purpose, calibration graphs of standard samples of quercetin, kaempferol and ellagic acid were previously constructed. Concentrations of calibration solutions were in the range of 0.002 – 0.03%. The prepared solutions were further chromatographed under the above conditions, determining the peak area. According to the results, the calibration graphs presented in fig.2–4 were built.

As can be seen in the figures, in the indicated range of standard concentrations of witness substances, the calibration dependence was linear, as evidenced by the values of the correlation coefficient, which in all cases amounted to 0.999 – 1.000.

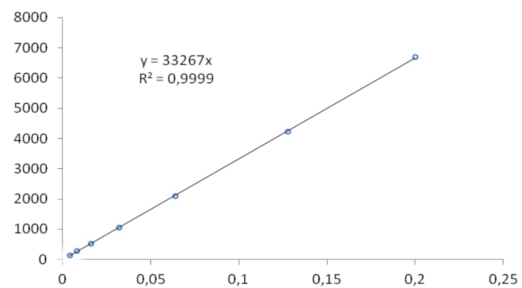

Fig.2. Calibration graph. Y-axis of the plot is peak area of kaempferol standard (mAU·s); X-axis of the plot is concentration, % w/v.

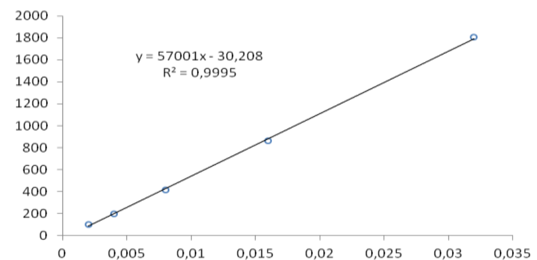

Fig.3. Calibration graph. Y-axis of the plot is peak area of quercetin standard (mAU·s); X-axis of the plot is concentration, % w/v.

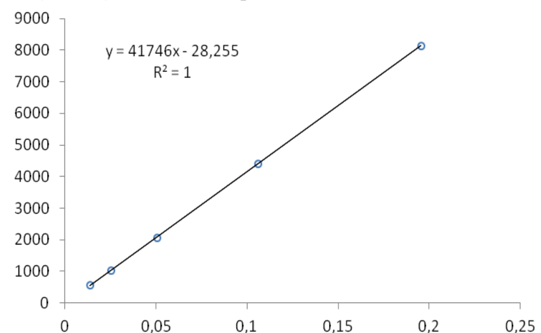

Fig.4. Calibration graph. Y-axis of the plot is peak area of ellagic acid standard (mAU·s); X-axis of the plot is concentration, % w/v.

It is known that in order to determine the content of aglycones in plant sources, it is necessary to preliminarily hydrolyze the extraction from plant materials to release them.

We used a fundamentally new approach, which consists in the extraction of biologically active compounds from *P. fruticosa* L. herb with ethyl alcohol containing an acid agent. In this case, extraction and hydrolysis of flavonoid glycosides occurs simultaneously. After completion of the extraction and hydrolysis process, the obtained extract is subjected to chromatography using liquid chromatography.

The optimal solvent and the ratio of raw material and extractant were previously selected to implement the methodology.

Ethanol in concentrations of 95%, 70% and 40%, with a hydrochloric acid content of 1%, was taken as an extractant. The resulting extracts from the feed were chromatographed under the conditions given above. The chromatogram of acidic alcohol extraction from *P. fruticosus* L. herb is shown in Fig.5.

Thus, aglycones were found in the hydrolysis products: quercetin, kaempferol, ellagic acid, and trace amounts of rhamnetin.

Analyzing the results presented in table II, we can conclude that the most suitable extractant for the quantification of aglycones of flavonoids and ellagic acid is ethyl alcohol 70%, acidified with hydrochloric acid.

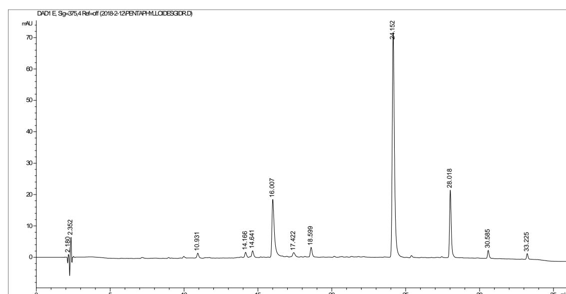

Fig.5. Chromatogram and UV spectra of polyphenols of *P. fruticosa* L. grass after acid hydrolysis.

Next, the optimal ratio of raw materials and extractant. Raw materials were extracted in ratios of 1:10; 1:25; 1:50; 1: 100 in relation to the extractant. The content of quercetin, kaempferol and ellagic acid in the obtained extracts was calculated by the formula (1):

$$x = \frac{S_x \times c_{st} \times V_{st} \times W_1 \times 100}{S_{st} \times m_x \times (100 - W)} \quad (1)$$

where:

S_x – average peak area of the determined aglycone calculated in 3 parallel observations of the test solution;

S_{st} – average peak area of standard of the corresponding aglycone calculated in 3 parallel observations of the comparison solution;

C_{st} – standard concentration of the corresponding aglycone, %;

m_x – sample weight of the test material, in grams;

W – volumetric flask volume;

W – loss in mass when drying the material, %.

The calculation results of the quantitative content of the corresponding aglycones are shown in table III.

A comparative analysis of the extracting capacity of ethyl alcohol in the concentrations used with the addition of hydrochloric acid is presented in table II.

According to the results presented in table III, the optimal ratio of raw materials and extractant is 1:50.

Thus, the technique developed by us consists in the following.

An analytical sample of the air-dried test material was ground to the size of particles passing through a sieve with openings of 1 mm in size. An exact sample of 2.0 g of the prepared material was transferred to a flask with a 100 ml capacity, 25 ml of 1% solution of hydrochloric acid in 70% ethyl alcohol was poured, attached to the reverse refrigerator and placed in a boiling water bath with an exposure time of 30 minutes. The flask was then cooled to room temperature and the contents filtered through a paper filter into a 100 ml volumetric flask. the Extraction was repeated three more times in the manner described above. The filtrate was collected in the same volumetric flask. The volume of the filtrate in the volumetric flask was brought to the mark by the same extractant.

TABLE II. RESULTS OF A COMPARATIVE ANALYSIS OF THE EXTRACTIVE ACTIVITY OF ALCOHOLS OF DIFFERENT CONCENTRATIONS IN THE EXTRACTION OF POLYPHENOLS OF P. FRUTICOSUS L.

Concentration of ethyl alcohol with hydrochloric acid, %	Quercetin content in raw, %	Kampferol content in raw materials, %	The content of ellagic acid in raw materials, %
95	0,26	0,08	0,63
70	0,335	0,1	0,86
40	0,26	0,02	0,34

TABLE III. THE RESULTS OF THE QUANTITATIVE DETERMINATION OF AGLYCONES IN THE GRASS OF P. FRUTICOSUS L. BY DIFFERENT CONCENTRATIONS OF ETHYL ALCOHOL, ACIDIFIED WITH HYDROCHLORIC ACID

The ratio raw materials-extractant	Quercetin content in raw materials, %	Kampferol content in raw materials, %	The content of ellagic acid in raw materials, %
1:10	0,25	0,05	0,49
1:25	0,31	0,074	0,56
1:50	0,335	0,1	0,86
1:100	0,334	0,098	0,87

The resulting extraction in an amount of 5 μl was subjected to chromatographic separation by method of High Performance Liquid Chromatography on a column, for example, Ascentisexpress C182,7μm × 100 mm × 4,6 mm, or similar under the conditions given above.

On the chromatogram of the test alcohol solution, the retention time of the peaks of quercetin, kaempferol, ellagic acid should coincide with the time of the retention of the peaks in the chromatograms of CO of quercetin, kaempferol (375 nm), ellagic acid (254 nm).

Notes. 1. *Preparation of standard solutions of quercetin, kaempferol, ellagic acid.* 10 mg of these COs were placed in volumetric flasks with a capacity of 100 ml, 80 ml of ethyl alcohol 95% was added, the solution volume was adjusted to the mark with the same solvent and mixed.

2. ***Preparation of the mobile phase A.*** 10 ml of formic acid was placed in a volumetric flask with a capacity of 1000 ml, the volume of the solution was adjusted with ultrapure water (HPLC) to the mark and mixed. The pH of the solution should be in the range of 2-3.

3. Preparation of the mobile phase B. Ethylene or methyl alcohol for chromatography is used for chromatography.

The quantitative determination of these components in the raw material was carried out by the absolute calibration method. Quercetin, kaempferol, and ellagic acid were used as standard.

To construct calibration graphs of 0.02 g (analytical sample), STANDARD was placed in 50 ml volumetric flasks, 10 ml of 95% ethyl alcohol was added, thoroughly shaken until complete dissolution, and adjusted to the mark with the same solvent (solutions A).

From solutions A, a series of calibration solutions was prepared, including 6 samples. For this, in each of 6 volumetric flasks with a volume of 25 ml, solutions A were transferred with a pipette in volumes: 2.5; 5.0; 7.5; 10.0; 12.5, 15.0 ml, the contents of the flasks were mixed and adjusted with ethyl alcohol 95% to the mark (solutions B).

5 μl of the obtained calibration solutions were introduced into the chromatograph and their peak areas were recorded. According to the results, graphs of the dependence of the peak area on the amount of the introduced substance were constructed.

The calculation of the content of the components was carried out on the basis of the constructed calibration graphs using the appropriate regression equations.

A validation assessment of the developed method was carried out according to the criteria: linearity, precision and correctness. The obtained results indicate the correctness of this technique.

IV. CONCLUSION

Thus, as a result of this study, the composition of the polyphenolic complex of the herb *P. fruticosus* L. was established, including the glycosides of quercetin, kaempferol, rhamnetin, proacyanidins, elagic acid and ellagotannins. A method has been developed for the quantitative determination of the sum of aglycones of grass polyphenols of *P. fruticosus* L., which is based on the principle of extraction with the simultaneous hydrolysis of glycosides of polyphenols to aglycones and their subsequent chromatographic determination by high performance liquid chromatography and quantification using absolute calibration.

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